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Award Number: W81XWH-04-1-0449

TITLE: Structure and Mechanism-Based Design of ErbB Receptor Inhibitors

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REPORT DATE: September 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-09-2007		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 SEP 2004 - 31 AUG 2007	
4. TITLE AND SUBTITLE Structure and Mechanism-Based Design of ErbB Receptor Inhibitors				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0449	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Daniel J. Leahy  E-Mail: dleahy@bs.jhmi.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  Johns Hopkins University School of Medicine Baltimore, MD 21205				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The HER4/ErbB4 receptor tyrosine kinase is inhibited by lapatinib (Tykerb™), and a crystal structure of the HER4/ErbB4 kinase domain complexed with lapatinib reveals an inactive conformation of the kinase and the specific molecular contacts made between HER4/ErbB4 and lapatinib. A crystal structure of the HER4/ErbB4 kinase domain in an active conformation reveals an asymmetric dimer contact virtually identical to an interaction observed for the related epidermal growth factor receptor. Mutagenesis studies demonstrate this dimer contact to be essential for normal kinase activation and show this activation mechanism to be a general feature of the ErbB family of receptor tyrosine kinases. An integral membrane form of the epidermal growth factor receptor has been expressed and purified and will form the basis for future structural and functional studies of the EGFR/ErbB family of receptors.					
15. SUBJECT TERMS HER4, ErbB4, HER2, ErbB2, EGFR					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER (include area code)

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## Introduction

The subject of this research was the ErbB family of receptor tyrosine kinases (RTKs), which includes the epidermal growth factor receptor (EGFR/ErbB1/HER1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Inappropriately activated forms of ErbBs are associated with many human cancers, and drugs targeting ErbBs have been approved for the treatment of breast (Trastuzumab, Lapatinib), colorectal (Cetuximab), head-and-neck (Cetuximab), and lung (gefitinib, erlotinib) cancers. The purpose of this research was to elucidate the molecular mechanisms governing ErbB activity in normal and disease states. How specific drugs target ErbB activity was also investigated, and a structural basis for the design of new therapeutics targeting ErbBs established. The scope of the research involved biochemical and X-ray crystallographic investigations of ErbB family members and active fragments of ErbB family members.

## Body

The work initially proposed for this award involved using the crystal structures of the extracellular domains of the EGF receptor (EGFR) and its homologs HER2/Neu/ErbB2, HER3/ErbB3, and HER4/ErbB4, which were recently determined in my lab and elsewhere, to design a new class of inhibitors of this family of receptors. These structures had shown that ligands bind to two separate surfaces in these receptors that are normally far apart in the absence of ligand. Binding ligand (e.g. EGF) requires a large conformational change in the receptor to bring these surfaces close together, and it is this conformational change that then leads to receptor dimerization and initiation of a signaling cascade through activation of a cytoplasmic tyrosine kinase. Our idea had been to create through mutagenesis a ligand that bound more tightly to one of the binding surfaces but not to the other. Theoretically, this ligand would bind to the receptor but not induce the conformational change needed to activate the receptor and thus serve as an inhibitor of the receptor.

As noted in previous annual reports, in the early stages of this project I learned of at least two companies and two academic labs that were pursuing similar if not identical strategies. After much deliberation, I decided not to duplicate their efforts but rather to redirect our energy to a different project that also involved structure-based design of ErbB inhibitors and maintained the spirit of the original proposal. Owing to unfamiliarity on my part with the procedures for altering the goals of a research project a revised statement of work was not submitted until last year, an oversight I regret.

The revised Statement of Work:

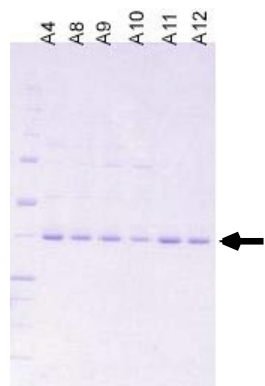
1. Determination of a crystal structure of the human HER2/ErbB2 kinase domain.
  - 1a. Express the human HER2/ErbB2 kinase domain using a baculovirus expression system.
  - 1b. Purify sufficient quantities of the HER2/ErbB2 kinase for structural studies.
  - 1c. Characterize the enzymatic properties and aggregation state of the purified HER2/ErbB2 kinase.
  - 1d. If not monodisperse, identify mutations or solution conditions that result in a soluble, monodisperse form of the kinase.
  - 1e. Crystallize and determine the X-ray structure of the HER2/ErbB2 kinase in native and inhibited states.
2. Determination of the crystal structure of the HER4/ErbB4 kinase region. The EGFR/ErbB1 kinase domain structure has been determined in many states and the HER3/ErbB3 kinase is inactive. By targeting the HER4/ErbB4 kinase as well as the HER2/ErbB2 kinase, we hope to elucidate general and specific features of the three active members of the ErbB receptor family (EGFR/ErbB1, HER2/ErbB2, HER4/ErbB4).
  - 2a. Express the human HER4/ErbB4 kinase domain using a baculovirus expression system.
  - 2b. Purify sufficient quantities of the HER4/ErbB4 kinase for structural studies.

- 2c. Characterize the biochemical properties and aggregation state of the purified HER4/ErbB4 kinase.
  - 2d. If not monodisperse, identify mutations or solution conditions that result in a soluble, monodisperse form of the kinase.
  - 2e. Crystallize and determine the X-ray structure of the HER4/ErbB4 kinase in native and inhibited states.
3. Purification and characterization of intact members of the ErbB family to allow comparison of activities of intact receptor tyrosine kinases with their isolated kinase domains.
    - 3a. Express an integral membrane form of human EGFR.
    - 3b. Purify sufficient quantities of this form of EGFR for structural studies.
    - 3c. Characterize the biochemical properties and aggregation state of the purified EGFR.
    - 3d. If not monodisperse, identify mutations or solution conditions that result in a soluble, monodisperse form of the kinase.
    - 3e. Crystallize and determine the X-ray structure of this form of EGFR in native and inhibited states.

### Aim 1 Results.

As noted in last year's annual report, a baculovirus expression system for the expression of the HER2/ErbB2 kinase domain (HER2KD) was developed, and HER2KD was purified to homogeneity (Figure 1). Purified HER2KD was shown to be active with a  $K_m$  of  $57 \pm 5$

micromolar and a  $k_{cat}$  of  $6.1 \pm 0.22 \text{ min}^{-1}$  for an optimal peptide substrate (Figure 2). Purified HER2KD was inhibited by lapatinib (Tykerb™). Unfortunately, expression levels of HER2KD were low, ~0.2 mg per liter of insect cell culture, and only ~25% of the expressed HER2KD was not aggregated as judged by size-exclusion chromatography. Purified HER2KD was also unstable, and the majority of unused sample precipitated within a few days of purification.

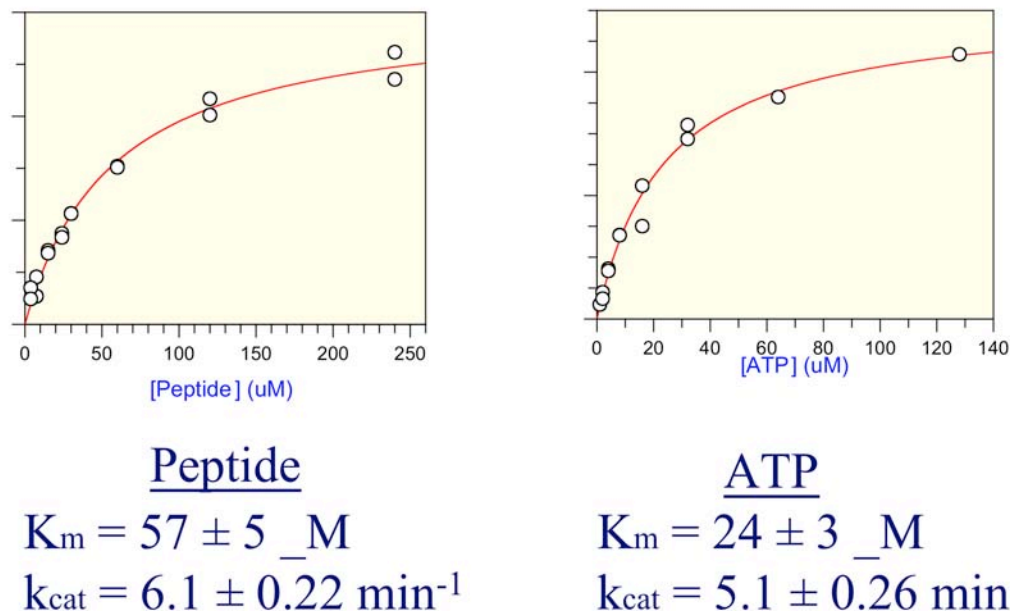


**Figure 1. SDS-PAGE analysis of purified HER2KD, which is indicated by the arrow.**

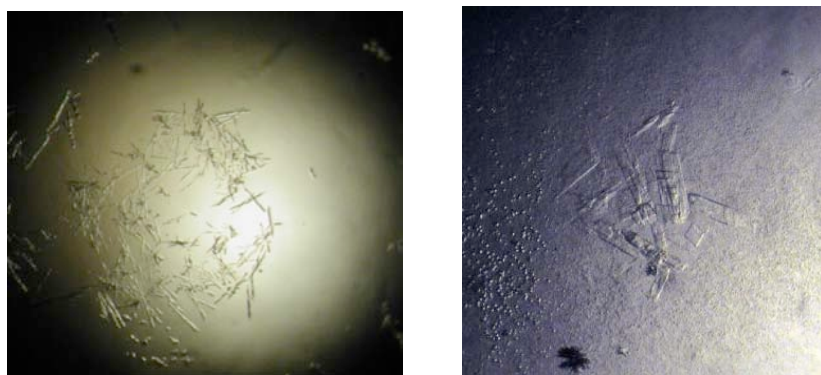
To address the solubility/aggregation problem, nine hydrophobic residues in HER2KD were identified that correspond to exposed hydrophilic residues in EGFR (Stamos et al., 2002). These nine sites in HER2 were mutated to the corresponding EGFR residue. The aim of this experiment was to produce a form of the HER2KD that would retain a native HER2KD enzymatic active site (none of the mutations is near the kinase active site) but would possess the favorable solubility and crystallization properties of the EGFR kinase domain. The variant form of the HER2KD (solHER2KD) was expressed and purified.

Unfortunately, the solubility properties of solHER2KD were not improved relative to wild type HER2KD. Small crystals of both HER2KD and solHER2KD were grown but extensive screening

of related conditions failed to result in crystals sufficiently large to assess their diffraction properties (Figure 3). Extensive screens of solute and buffer conditions (pH, detergents, glycerol, salt, etc.) also failed to identify conditions in which either HER2KD or solHER2KD exhibited improved solubility and stability. The appearance of diffraction-quality crystals of HER4KD led us to focus on studies of that domain, but we plan to build on our current experience and return to studies of HER2KD owing to its established importance as a drug target. To overcome the problems of our current studies, our future work with HER2KD will involve introducing additional “solubilizing” mutations and testing HER2KD proteins with different N- and C-termini for better solubility.



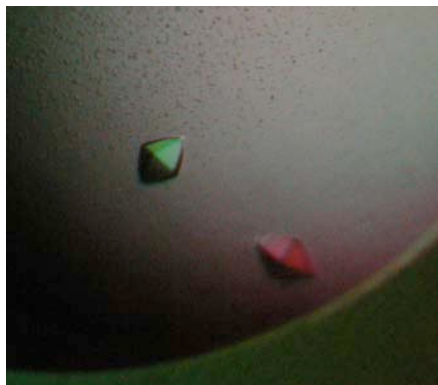
**Figure 2.  $K_m$  and  $k_{cat}$  for HER2KD for both an optimal peptide substrate and ATP.**



**Figure 3. Crystals of HER2KD (left) and solHER2KD (right).**

## Aim 2 Results.

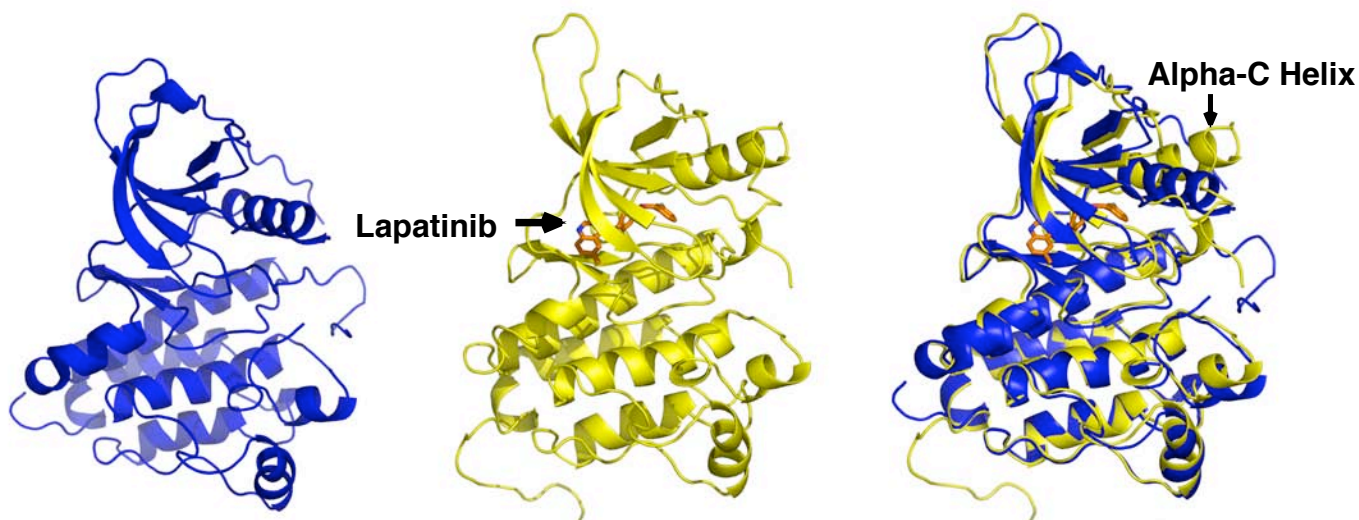
To investigate shared and specific features of kinase activation among ErbBs, a baculovirus expression system was developed for the HER4/ErbB4 kinase domain (HER4KD). HER4KD was purified, shown to be catalytically active, shown to be inhibited by lapatinib, and crystallized in the presence and absence of lapatinib (Figure 4). Lapatinib (Tykerb™) is an inhibitor of ErbB kinases that was recently approved for the treatment of HER2-overexpressing breast cancer (Geyer et al., 2006).



The structures of both active and lapatinib-inhibited forms of HER4KD have now been determined by X-ray crystallography (Figure 5). Refinement of the active HER4KD structure is complete, and refinement of the HER4KD-lapatinib complex is in its final stages (Table 1). HER4KD in the absence of lapatinib exhibits the characteristic structural features of an active kinase, including the disposition of the activation loop and the alpha-C helix, and HER4KD complexed with lapatinib exhibits the characteristic structural features of an inactive kinase (Figure 5).

**Figure 4. Crystals of the HER4 kinase domain. The longest dimension of the “diamond” is ~0.05 mm.**

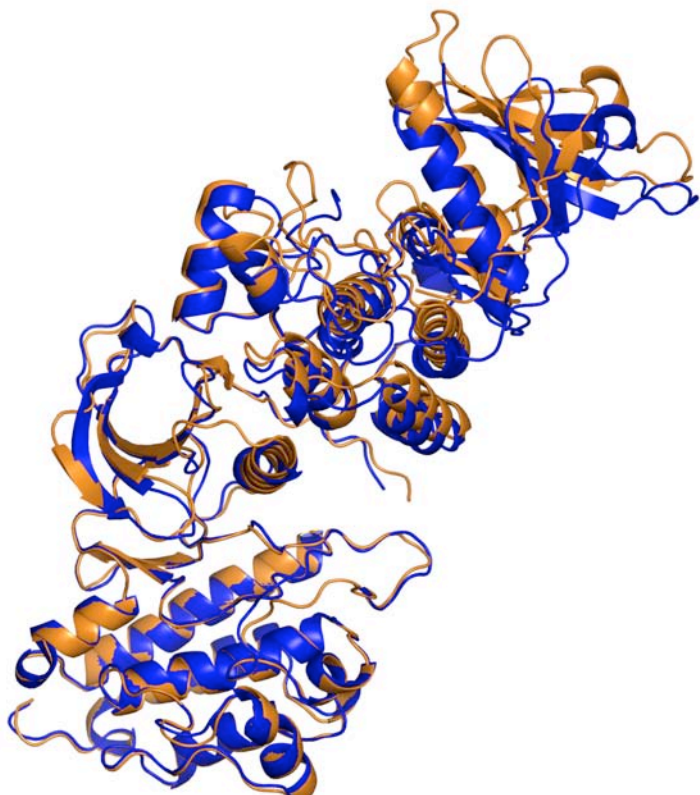
Analysis of the structures is in its early stages as the refinement is not yet complete, but perhaps the most exciting aspect of the active HER4KD structure is that it adopts an asymmetric dimer conformation that is virtually identical to the asymmetric dimer observed for the EGFR kinase domain by Zhang et al. (2006) (Figure 6). The EGFR kinase asymmetric dimer is believed to form following ligand-induced dimerization of the EGFR extracellular regions and be essential to activation of the kinase activity. Our observation of the identical asymmetric dimer in an active form of



**Figure 5. Ribbon diagrams of HER4KD. Left: HER4KD in active form; Middle: inactive lapatinib-inhibited form of HER4KD, lapatinib shown in orange; Right: superposition of inactive (blue) and active (yellow) forms of HER4KD. The alpha-C helix is indicated.**



HER4KD provides unequivocal evidence for the existence and generality of this mechanism for regulation of ErbB kinase activity.



**Figure 6. Superposition of the HER4KD asymmetric dimer (blue) with the EGFR kinase domain asymmetric dimer (brown) (Zhang et al. (2006)).**

of Pennsylvania, and they have shown that structure-based mutations in HER4 at the asymmetric dimer interface impair ligand-induced activation of intact HER4 on the surface of cells. A manuscript describing the HER4KD structure, enzymatic, and functional properties is being prepared and will be submitted for publication in October of 2007.

Several crystal structures of the EGFR kinase domain have been reported (Stamos et al., 2002; Wood et al., 2004; Zhang et al., 2006), and the inhibitory properties of lapatinib with respect to EGFR and HER4 differ somewhat. Once refinement of the HER4KD/lapatinib co-crystal structure is complete, an analysis of the contacts between lapatinib and the two ErbB kinases is planned to identify structural features that may underlie the different responses to lapatinib and guide design of either more specific or more effective ErbB kinase inhibitors.

As also noted by Zhang et al. (2006), the asymmetric dimer interface offers a new target for small molecule inhibitors of ErbB kinase activity. Early analysis of the HER4KD asymmetric dimer interface suggests that the interface is so highly conserved that any molecules targeting this interface will not be ErbB specific.

We have established a collaboration with the lab of Mark Lemmon at the University

**Table 1.** Crystallization, data collection and refinement statistics for HER4KD crystals

Crystal	ErbB4 Kinase domain	ErbB4 kinase domain complexed with lapatinib
condition	15%PEG3350, 0.1 M Tris, pH 8.5	4.3M NaCl, 0.1 M Hepes, pH 7.0, 2%TFE
Spacegroup	P3 <sub>2</sub>	P6 <sub>1</sub>
Unit cell (Å)	a=b=86.7, c=120.0	a=b=102.6, c=185.1
Resolution (Å)	30.0-2.50 (2.59-2.50)	30.0-2.90(3.00-2.90)
Number of molecules per ASU	3	2
Solvent content	47.5%	68%
Data completeness (%)	100 (100)	99.9 (99.6)
redundancy	2.6	4.8
R <sub>sym</sub> (%)	10.7 (45.7)	6.2 (51.4)
I/σI	8.8 (1.9)	24.5 (2.8)
Refinement		
Phaser Search model	1M14	1XKK
R/R <sub>free</sub> (%)	20.2 / 25.5	25.0 / 29.5
Number of protein atoms	6846	
Number of non-protein/solvent atoms	1	
Number of solvent atoms	173	
Ramachandran plot		
Most favored (%)	89.6	
Additional allowed (%)	9.9	
Generously allowed (%)	0.5	
Rmsd bond length (Å)	0.01	
Rmsd bond angle (°)	1.3	
Mean B-value	19.2	

### Aim 3 Results.

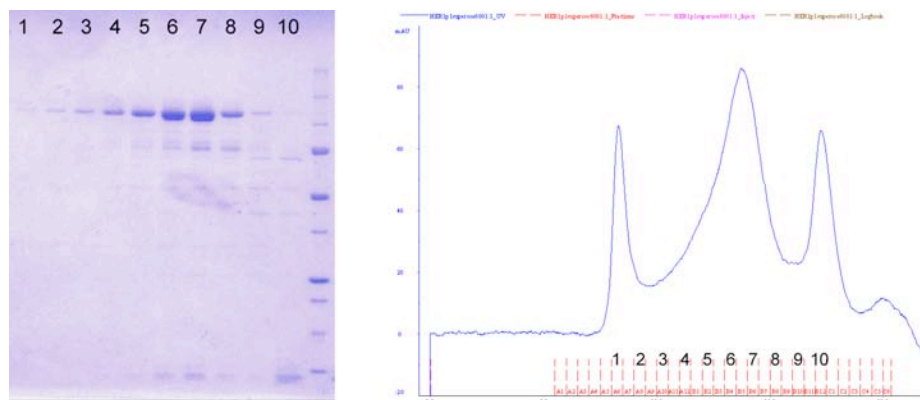
To relate structural and biochemical results obtained with the isolated kinase domains of ErbBs, we have attempted to produce intact forms of ErbBs. ErbBs consist of an ~630 amino acid extracellular ligand-binding region followed by a single membrane-spanning region, a cytoplasmic tyrosine kinase, and a C-terminal region of ~230 amino acids that harbors several sites of tyrosine phosphorylation (Burgess et al., 2003). We expressed full-length forms of both EGFR and HER2/ErbB2 in both Chinese Hamster Ovary (CHO) and Human Embryonic Kidney 293 (HEK293) cells. In our early experiments, the expressed EGFR and HER2 were invariably partially proteolyzed during purification at a site between the kinase domain and the C-terminal tail. We thus introduced a stop codon following the kinase domain to express a truncated form of the receptors that was uniform in size. Earlier reports had indicated that the truncated ErbBs retained ligand-inducible kinase activity (Chinkers and Brugge, 1984), and we verified that our truncated EGFR (tEGFR) indeed retained both ligand binding and kinase activity.

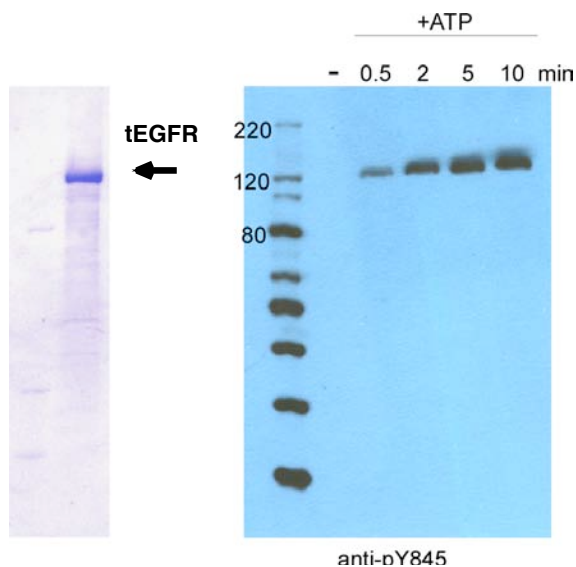
Expression levels of tEGFR were always several-fold greater than those of HER2/ErbB2, and we chose to focus on tEGFR for this reason and the fact that we could monitor its ligand-inducible kinase activity (HER2/ErbB2 has no ligand). In the midst of preparing and selecting stably-transfected tEGFR cells for high expressers, we noted publication of a transient-transfection method that greatly reduced the cost and turnaround time of protein expression in mammalian cells (Aricescu et al., 2006). We obtained the appropriate vectors, modified them to suit our needs, and have now switched to expressing tEGFR in transiently-transfected HEK293 cells with modified glycosylation properties that make glycosylation simpler to remove (Reeves et al., 2002).

We initially expressed tEGFR with a hexa-histidine tag to enable rapid affinity purification with Nickel-NTA beads but found that the efficiency of this method was poor. A hybridoma cell line secreting a monoclonal anti-EGFR antibody that competes for EGFR binding with EGF was then obtained, and an affinity purification procedure developed using this antibody coupled to a solid matrix. Cell lysates are poured over this modified matrix, and bound tEGFR eluted with excess EGF (in the detergent dodecylmaltoside). This procedure worked well and led to the ability to purify ~0.6 mg of tEGFR from ~10 liters of transfected cells (Figure 7).

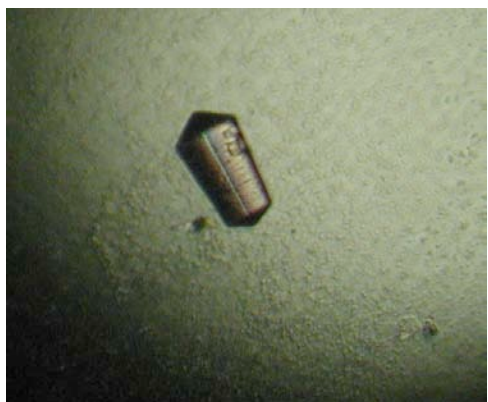
Purified tEGFR is complexed with EGF following elution from the antibody-affinity column, but it is not phosphorylated until addition of ATP (Figure 8). Autophosphorylation of purified tEGFR

**Figure 7. Gel-filtration chromatography trace (right) with tEGFR in the peak in fractions 5-8. SDS-PAGE analysis of gel-filtration chromatography fractions of tEGFR (left).**





**Figure 8. Coomassie-blue stained SDS-PAGE analysis of purified and concentrated tEGFR (left). Anti-phosphotyrosine Western blot of tEGFR at specified times following addition of ATP. The antibody in this case is specific for Tyr 845 of EGFR.**



**Figure 9. tEGFR crystal. The longest dimension is ~0.1 mm.**

demonstrates that the kinase activity of tEGFR is intact. Crystallization trials were initiated with purified tEGFR, and small paracrystalline objects were observed in multiple conditions. Adjustment of conditions has results in formation of larger crystals of tEGFR (Figure 9), but preliminary X-ray diffraction analysis using rotating anode radiation has failed to show diffraction. To accommodate the need for large cell culture volumes to produce the amounts of tEGFR needed for biochemical and X-ray diffraction experiments, we have recently expanded the capacity of our tissue culture facilities to handle 5-10 liter preparations. We are in the process of gearing up production to ensure a reliable supply of purified tEGFR for continued crystallization experiments and determination of  $K_m$  and  $k_{cat}$  for comparison with values obtained for the isolated EGFR kinase domain.

We are holding off publication of our results with tEGFR until we are either successful with our crystallization experiments or all reasonable alternatives have been exhausted. If our crystallization efforts ultimately prove unsuccessful, our expression and purification of tEGFR coupled with unequivocal

demonstration of autophosphorylation at Tyr 845 (Figure 8) will be prepared for publication. EGFR is known to be phosphorylated at Tyr 845, but this phosphorylation is believed to be mediated by Src (Wu et al., 2002), and demonstration of autophosphorylation of EGFR at Tyr 845 has implications for the regulation of EGFR activity and downstream signaling.

## Key Research Accomplishments

- Expression, purification, and enzymatic characterization of the HER2/ErbB2 kinase domain (crystallization efforts unfortunately proved unsuccessful).
- Expression, purification, crystallization, and determination of the crystal structure of the HER4/ErbB4 kinase domain in active and inactive forms. This work demonstrated that the “asymmetric dimer” mechanism of ErbB kinase activation observed for the EGF receptor is also utilized by HER4/ErbB4, which unequivocally established this mechanism as a key and general in ErbB family members.
- Heterologous expression and purification of several hundred micrograms of an integral membrane form of the EGF receptor for structural and biochemical studies.

## Reportable Outcomes

- A MS describing the HER4KD structural and functional studies is being prepared.
- Expression/purification of tEGFR and demonstration of autophosphorylation of tEGFR at Tyr 845 will be published following completion of structural studies of tEGFR.

## Conclusion

At present, the primary result of the supported research is the demonstration by X-ray crystallography of the same asymmetric dimer conformation in the active HER4 kinase domain that was observed for the EGFR kinase domain. This observation unequivocally establishes the asymmetric dimer as a key and general feature of ErbB kinase activation. Determination of the structure of the HER4 kinase domain in an inactive conformation with lapatinib (Tykerb™) also establishes a structural basis for understanding the differential effects of this pan-ErbB inhibitor on different ErbBs as well as the design of related inhibitors with increased ErbB specificity.

Secondly, we have produced an active form of the HER2 kinase domain and developed considerable experience working with this molecule. Although our attempts to crystallize the HER2KD have proven unsuccessful to date, the clinical importance of the HER2 kinase as a validated drug target motivates us to build on our experience and continue these studies. We plan to create additional “solubilizing” mutations in HER2KD, test the effects of longer and shorter N- and C-termini, and co-express HER2KD with potential binding partners (e.g. the HER3 kinase domain and Mig6) that may improve its solubility properties.

Thirdly, we have produced and purified an active integral membrane form of the EGF receptor. We have shown that this purified EGFR is capable of auto-phosphorylation at Tyr 845, which EGFR was not believed to do. As Tyr 845 is in the EGFR kinase “activation loop”, this phosphorylation is likely to be an important feature regulating the EGFR kinase activity. Our

purified EGFR is available in sufficient quantities and sufficient homogeneity to enable crystallization trials of this molecule, and preliminary crystals have been grown. No diffraction has been observed from these crystals, but their presence is a hopeful sign that better crystals may be obtained through variation of crystallization conditions.

So what? Structural and structure-based functional studies are essential to understanding the molecular mechanisms regulating the activity of growth factor receptors in both normal and disease states. Such studies also provide key insight into the mechanism and specificity of drugs targeting these receptors, inappropriately activated forms of which are associated with many human cancers. Our results firmly establish the “asymmetric dimer” of ErbB kinases as a key feature of ErbB activation, which introduces a new interface to target with ErbB inhibitors. We have also determined the structure of the approved breast cancer drug lapatinib in complex with the HER4/ErbB kinase domain, which gives insight into its specificity for ErbB kinases and how related compounds with new inhibitory properties may be designed. Finally, the holy grail of molecular studies of receptor tyrosine kinases is to understand how extracellular ligand binding information is transmitted across the membrane to activate the cytoplasmic kinase domain. By expressing and purifying near milligram amounts of an active, deglycosylated integral membrane form of EGFR we have taken an important first step towards structural and functional studies addressing this key issue.

### **Salaries Supported Wholly or in Part by this Award**

Dan Leahy (P.I.)  
Patti Longo (Technician)  
Aruna Satyamurthy (Postdoc)  
Chen Qiu (Postdoc)

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